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IMMUNOCHEMICAL COMPARISON OF β-LACTOGLOBULINS

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β-Lactoglobulin from the domestic cow (*Bos taurus*) is among the best characterized of proteins. Four genetic variants (A, B, C, D) of it have been discovered (1–3). In addition, β-lactoglobulins from the goat (*Capra hircus*), sheep (*Ovis aries*) and buffalo (*Bubalus bubalis*) have been isolated and shown to be homologous to the bovine protein by similarity of amino acid content (4–9) and cross reaction with anti-bovine β-lactoglobulin (10). For brevity in this paper we designate β-lactoglobulin as Lg, the bovine, goat, sheep and buffalo proteins as Lgb, Lgc, Lgo, and Lgbu respectively, and the bovine genetic variants as Lgb-A, Lgb-B, Lgb-C and Lgb-D.

Gough and Jenness (11) investigated the immunologic relationship between Lgb-A and Lgb-B, using the quantitative precipitin test, analysis for cross reaction, immunoelectrophoresis and Ouchterlony double diffusion. The two genetic variants were shown to be identical by these criteria. De Weer *et al.* (12) compared Lgb-A and Lgb-B by Ouchterlony double diffusion and immunoelectrophoresis and reached the same conclusion. Saperstein (13) and Crawford and Grogan (14) have shown that antisera to Lgb react with goat's milk.

Johke *et al.* (10) found that antiserum to Lgb reacted with purified Lg's from the milks of the goat, sheep and water buffalo, but not with non-ruminant milks in Oudin and Ouchterlony diffusion tests. They also found that anti-Lgb reacted more strongly with the Lg's from sheep and goat than with Lgb itself, by Oudin diffusion. Lyster *et al.* (15) found also that anti-Lgb reacted with the Lg in the wheys of 17 ruminant species studied, but no reaction was obtained from wheys of 3 non-ruminant artiodactyls and 5 non-artiodactyls.

The present paper deals with the relationship between the several Lgb variants, and between Lgb and other artiodactyl Lg's, as determined by several immunologic techniques: Ouchterlony double diffusion, the quantitative precipitin test, complement fixation (C'F), and microcomplement fixation (MC'F). In addition, these techniques were used to examine the effect of certain chemical modifications on the antigenicity of the Lgb molecule. These modifications were: hydrolysis of the C-terminal -His-Ile sequence of Lgb-B by carboxypeptidase A to produce a modified Lgb-B (Lgb-B-mod), crystallization of Lgb-A with 2 moles of sodium dodecyl sulfate (Lgb-A-SDS) and reaction of Lgb-B with Ellman's reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (16) to block the 2 sulfhydryl groups of the molecule (Lgb-B-DTNB).

MATERIALS AND METHODS

β-Lactoglobulins. Lgb-A, B and C were obtained by crystallization from typed milks (1). Lgc was prepared according to Askonas (17). Lgb-D, Lgbu and Lgo were gifts. Lgb-B-mod was crystallized according to the procedure of Greenberg and Kalan (18). Lgb-A-SDS was prepared according to McMeekin *et al.* (19). Lgb-B-DTNB was prepared by reacting Lgb-B with a five molar excess of DTNB for 24 hr, in the dark, at room temperature, pH 7.6. Excess reagent was removed by dialysis against distilled water, changed three times daily for 2 days, and the dialyzed solution was freeze-dried. The Lgb-B-DTNB gave no further reaction with DTNB. Upon acrylamide gel electrophoresis at pH 8.6 both Lgbu and Lgb-D showed the presence of minor impurities. All others appeared to be homogeneous.

Antisera. Antisera against Lgb-B, Lgb-B-mod and Lgc were made in New Zealand White rabbits (20). Two lots of anti-Lgb-B were obtained, designated I and II. Lot I was pooled antiserum from four rabbits; lot II was pooled from two rabbits.

Guinea pig complement and anti-sheep red blood cell hemolysin was obtained from Baltimore Biological Laboratory. Sheep red blood cells were

obtained fresh weekly and stored at 0 to 5°C in Alsever's solution until used.

Ouchterlony test. This test was performed in 1% Noble Agar (Difco Laboratories) in the modified barbital buffer described by Campbell *et al.* (21). The center well was filled with anti-Lgb-B, lot I. In the surrounding wells were placed the several β-lactoglobulins.

Quantitative precipitin test. This was performed as described by Gough and Jenness (11). Anti-Lgb-B, lot I, 1:5 dilution, was used in three experiments to compare Lgb-B with Lgb-A, C, and D, with goat and sheep Lg's, and with Lgb-B-mod. Lgb-B and Lgb-B-mod were also compared vs. anti-Lgb-B-mod and Lgb-B and goat and sheep Lg's were compared vs. anti-Lgc.

Complement fixation and micro-complement fixation. The complement fixation technique (21) was used employing a 1:60 dilution of anti-Lgb-B, lot I. The highly sensitive micro-complement fixation technique of Wasserman and Levine (22) was used to further extend the comparative study. Lgb-A, C, D, goat, sheep and buffalo Lg's, Lgb-B-mod and Lgb-A-SDS were compared to Lgb-B using anti-Lgb-B, lots I and II at dilutions of 1:250 and 1:2000 respectively. Lgb-B-DTNB was compared to Lgb-B with antiserum II only.

RESULTS

Ouchterlony test. Double diffusion showed Lgb-A, B, C, D, Lgc, Lgo, Lgbu and Lgb-B-mod to be identical (Fig. 1).

Quantitative precipitin test. This showed the bovine variants to be identical against anti-Lgb-B (Fig. 2). Goat and sheep Lg's gave less precipitate than Lgb-B when tested against anti-Lgb-B (Fig. 3);[1] these three proteins also differed when tested against anti-Lgc (Fig. 4). Lgb-B and Lgb-B-mod were identical when tested against anti-Lgb-B and anti-Lgb-B-mod (Fig. 5).

Complement fixation and microcomplement fixation. In the complement fixation test Lgbu, Lgb-D and Lgb-B-mod were indistinguishable from Lgb-B (Fig. 6). Lgb-C bound slightly less complement than Lgb-B but perhaps the difference is not significant. Lgb-A appeared to differ significantly from Lgb-B. Again, goat and sheep Lg's were readily distinguishable from Lgb-B.

The micro-complement fixation test was found to be the most sensitive of the immunologic tests employed. Lgb-A, C, D, Lgc and Lgo could be distinguished from Lgb-B, and greater differences were found than with C'F (Figs. 7, 8). This highly sensitive technique could not, however, distinguish Lgb-B-mod, Lgb-B-DTNB and Lgbu

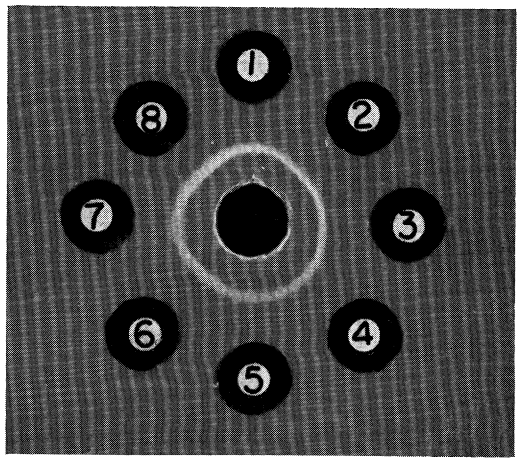

Figure 1. Ouchterlony double diffusion test. Diffusion of anti-Lgb-B vs. *1*, Lgc, *2*, Lgb-B, *3*, Lgo; *4*, Lgb-A, *5*, Lgb-C; *6*, Lgb-D; *7*, Lgb-B-mod; *8*, Lgbu. Developed for 72 hr.

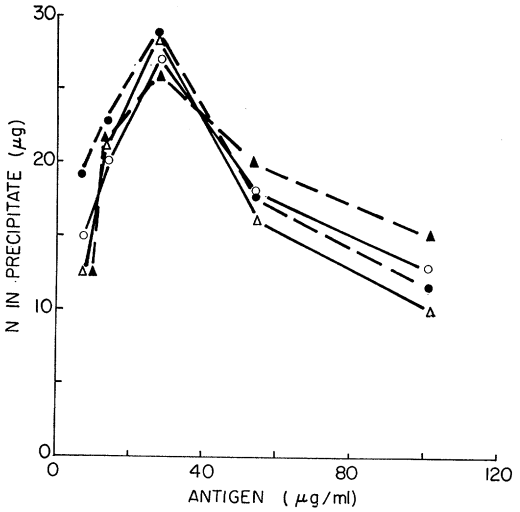

Figure 2. Quantitative precipitin test of anti-Lgb-B vs. Lgb-A (●), Lgb-B (○), Lgb-C (△), Lgb-D (▲).

[1] The statement in our abstract (Fed. Proc. *24:* 419, 1965) that goat β-lactoglobulin could not be distinguished from bovine β-lactoglobulin by quantitative precipitin tests is in error.

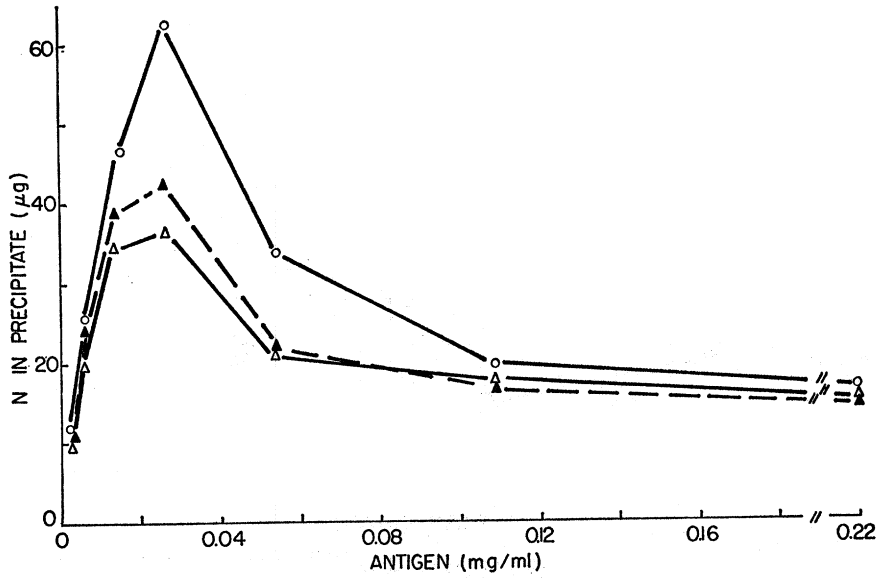

Figure 3. Quantitative precipitin test of anti-Lgb-B vs. Lgb-B (○), Lgc (△), Lgo (▲)

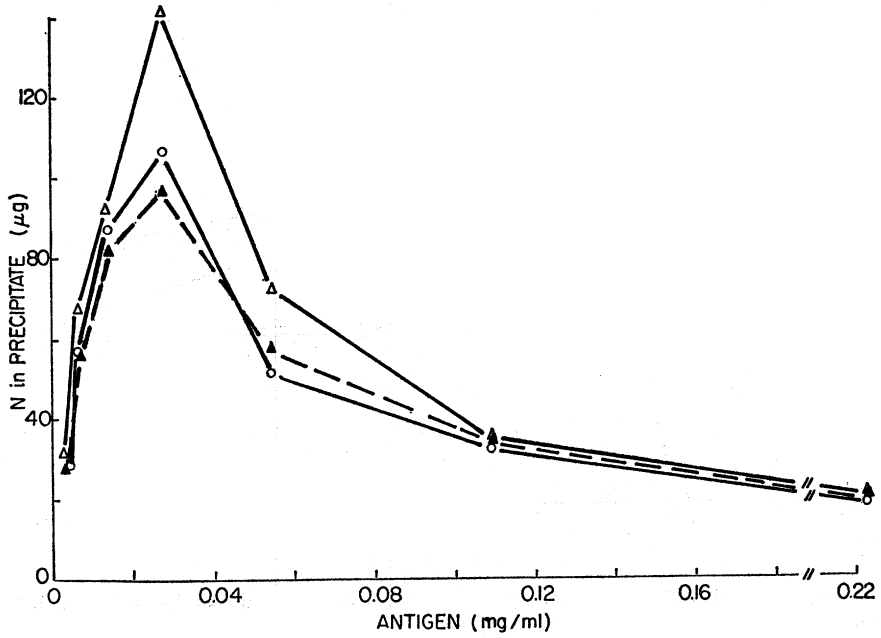

Figure 4. Quantitative precipitin test of anti-Lgc vs. Lgc (△), Lgb-B (○), and Lgo (▲)

from Lgb-B or Lgb-A-SDS from Lgb-A (Figs. 8 to 10). Consistent results were obtained with lots I and II of anti-Lgb-B.

DISCUSSION

The experiments described herein show the greater sensitivity of micro-complement fixation over other techniques employed. Its advantages of great sensitivity and use of extremely small quantities of antiserum, have been pointed out in studies of other homologous antigen systems, such as the hemoglobin variants (23) and the primate serum albumins (24). Micro-complement fixation enables one to define the degree of

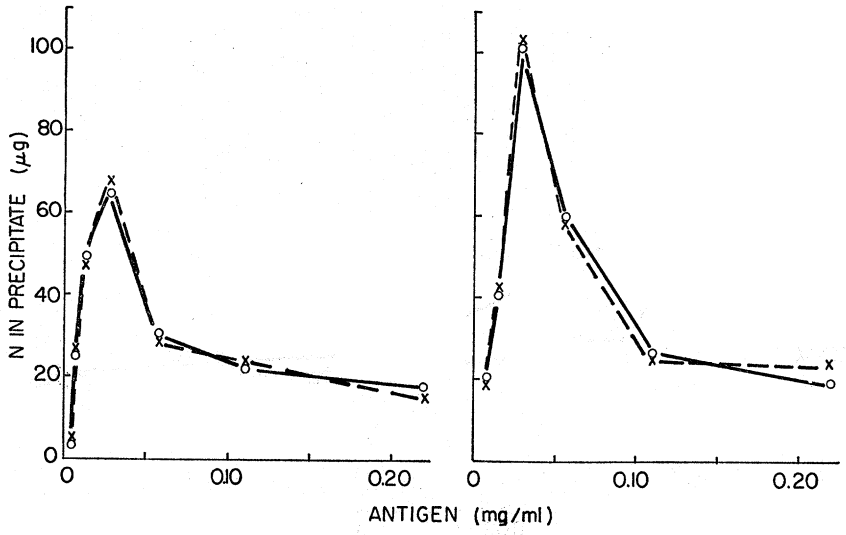

Figure 5. Quantitative precipitin test. *Left*, anti-Lgb-B vs. Lgb-B (○) and Lgb-B-mod (×). *Right*, anti-Lgb-B-mod vs. Lgb-B (○) and Lgb-B-mod (×).

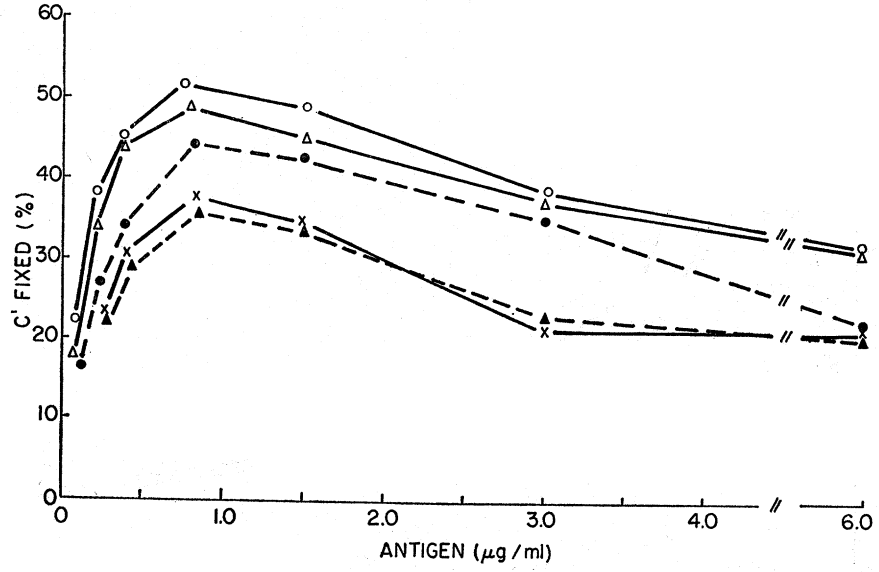

Figure 6. Complement fixation test. Anti-Lgb-B (1:60 dilution) vs. Lgb-A (●), Lgb-C (△), Lgc (×), Lgo (▲), and Lgb-B, Lgb-D Lgb-B-mod, Lgbu (○).

homology in the absence of information about amino acid composition and sequence. Antigens such as Lgb-A, B, C, D, which are indistinguishable by double diffusion and quantitative precipitin techniques may be distinguished by complement fixation tests particularly the microtest.

Lgb-A differs from Lgb-B by Val/Ala and Asp/Gly substitutions (4, 5); Lgb-C differs from Lgb-B by a Gln/His substitution (25); the amino acid composition of Lgb-D has not yet been published. From the data presented herein, it must be concluded that the amino acid differences between Lgb-A, B, C, and D either lie within the antigenic sites or close enough to affect them. On the other hand, chemical modifications of the

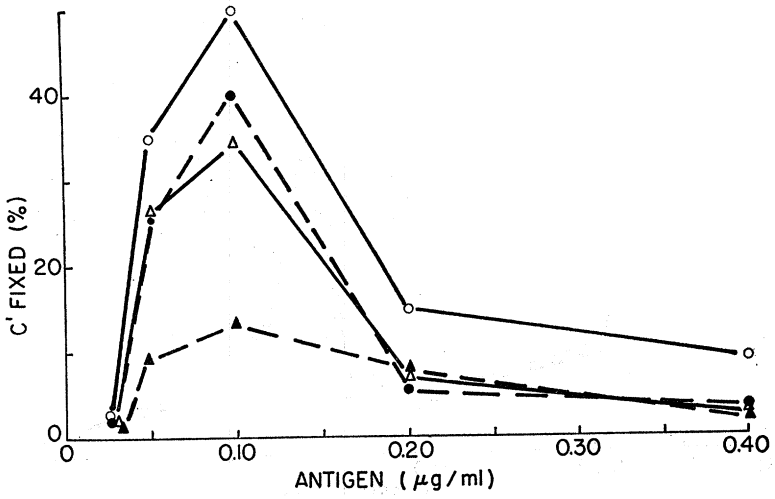

Figure 7. Micro-complement fixation test of anti-Lgb-B (1:250 dilution) vs. Lgb-B (○), Lgb-A (●) Lgb-C (△), and Lgb-D (▲).

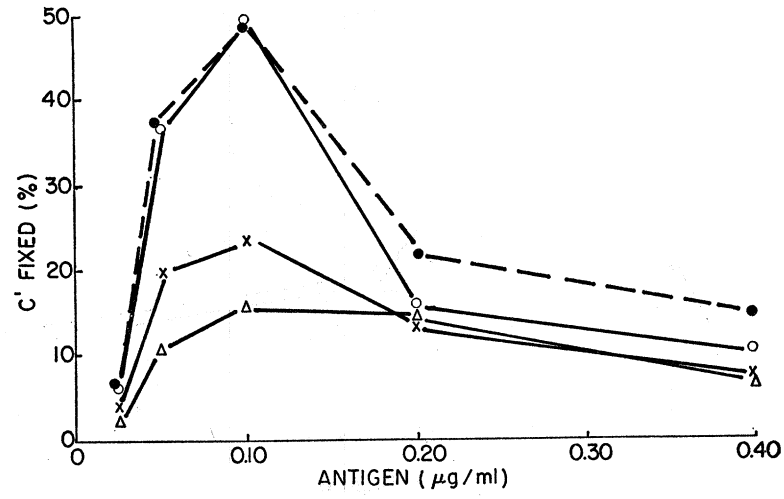

Figure 8. Micro-complement fixation test of anti-Lgb-B (1:250 dilution) vs. Lgb-B (○), Lgbu (●), Lgc (×), and Lgo (△).

Lg molecules, such as cleavage of the two C-terminal residues (Lgb-B-mod), crystallization with SDS (Lgb-A-SDS) and preparation of a DTNB blocked derivative (Lgb-B-DTNB) do not affect the antigenic sites. Lgb-B-mod has been shown to be identical to Lgb-B insofar as secondary configuration and tertiary structure are concerned (18).

If one takes Lgb-B as the prototype of Lgb, and compares its cross reactivity with the Lg's of other species, Lgbu appears identical to it by all tests employed, and goat and sheep Lg's antigenically distinct, except by Ouchterlony double diffusion. The amino acid composition of Lgbu has been reported identical to that of Lgb-B of the closely related cow (9). The Lg's of the more distantly related goat and sheep have amino acid compositions which differ from that of the cow Lg's (7, 8); these amino acid differences evidently affect the antigenic determinants of these molecules.

Lgo is reported to occur in two genetic variants

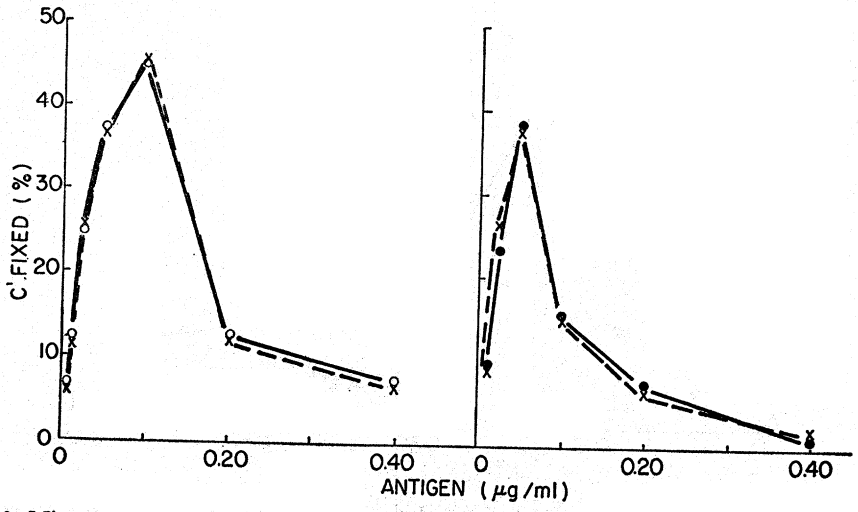

Figure 9. Micro-complement fixation test. *Left*, anti-Lgb-B (1:250 dilution) vs. Lgb-B (○) and Lgb-B-mod (×). *Right*, anti-Lgb-B (1:250 dilution) vs. Lgb-A (●) and Lgb-A-SDS (×).

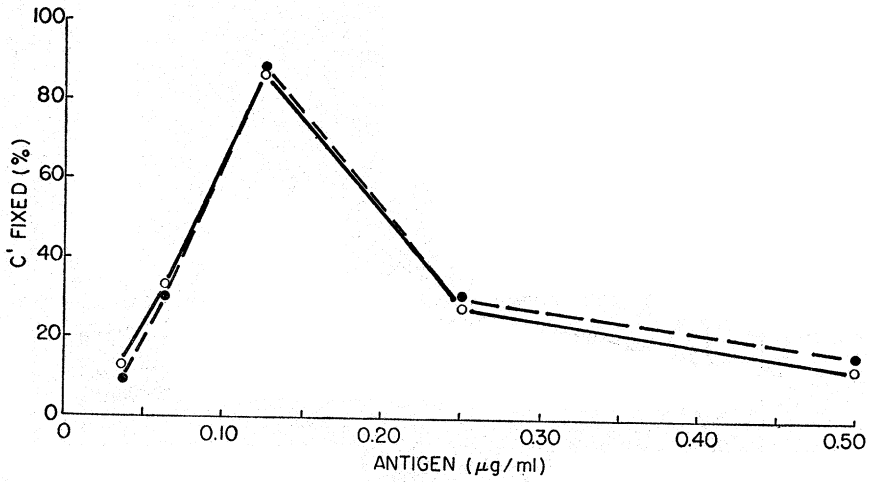

Figure 10. Micro-complement fixation test of anti-Lgb-B (antiserum II, 1:2000 dilution) vs. Lgb-B (○) and Lgb-B-DTNB (●).

(8). It was not possible for us to determine whether the sample of Lgo with which we worked contained one or both genetic variants.

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SUMMARY

The genetic variants of cow β-lactoglobulin are indistinguishable by Ouchterlony double diffusion and the quantitative precipitin test. They are distinguishable from each other by complement and micro-complement fixation tests.

Goat and sheep β-lactoglobulins are identical to the cow β-lactoglobulin variants by the Ouchterlony test, but are distinguishable by quantitative precipitation, complement and micro-complement fixation.

Chemical modification of cow β-lactoglobulin, such as cleavage of the -His-Ile C-terminal

sequence of the B variant, crystallization of the A variant with 2 moles of SDS and blocking of the -SH groups of the B variant with DTNB, do not produce molecules which are immunologically distinguishable from the parent molecule.

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